



APPENDIX B



Cloning of cDNA Encoding a Putative Chemoattractant Receptor

CHRISTER OWMAN,*¹ CHRISTER NILSSON,* AND STEPHEN J. LOLAIT†

*Section of Molecular Neurobiology, Department of Physiology and Neuroscience, Wallenberg Neuroscience Center, University of Lund, S-22362 Lund, Sweden; and †Laboratory of Cell Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20892

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Based on polymerase chain reaction (PCR) utilizing degenerate primers directed to the second and sixth transmembrane domains of several G-protein-coupled neurotransmitter receptors and screening of a human B-lymphoblast cDNA library, we isolated a cDNA whose predicted amino acid sequence shows considerable homology with human chemoattractant receptors, e.g., 30% overall identity with the C5a anaphylatoxin receptors. The coding region consists of 1056 bp corresponding to 352 amino acid residues and giving an approximate molecular weight of 43 kDa. Northern blot analysis showed hybridizing transcripts in spleen, thymus, and lymph nodes, as well as in bone marrow and peripheral blood leukocytes. Message was also found in lymphoid tumor cell lines. Chromosome mapping with FISH/DAPI technique showed the corresponding gene to reside on human chromosome 14q11.2-q12. In accordance with the Genome Database Nomenclature the receptor was designated *CMKRL1* ("chemoattractant receptor-like 1"). Stably transfected mammalian cells (CHO cells and LVP2.0Zc reporter cells) expressing high levels of corresponding receptor RNA were analyzed for changes in cAMP concentration and cellular calcium fluxes. Chemokines tested to date (GRO- α , MCP-1, MCP-3, MIP-1 α , MIP-1 β , C5a, RANTES, and LTB $_4$) have failed to elicit any reproducible response. Although the ligand for *CMKRL1* could thus not be identified among chemotactic peptides, the high expression in lymphoid cells and tissues suggests that the receptor may function in the regulation of the inflammatory system. © 1996 Academic Press, Inc.

INTRODUCTION

The superfamily of G-protein-coupled membrane receptors receives signals that show great variety in chemical structure and originate from a large number

of sources (Watson and Arkinstall, 1994). Recent rapid development in the understanding of the molecular mechanisms underlying the functions of these receptors is to a large extent based on the cloning and structural analysis of cDNA or genes encoding the receptors, not least the receptors receiving signals from neurotransmitter amines and peptides. Such work was pioneered by the groups of Lefkowitz (cf. Dixon *et al.*, 1986), Numa (cf. Kubo *et al.*, 1986), and Nakanishi (cf. Masu *et al.*, 1987).

In an attempt to identify cDNAs encoding new G-protein-linked receptors we used degenerate primers directed toward conserved regions of known (neurotransmitter) receptors in low-stringency PCRs (Libert *et al.*, 1989) to generate receptor DNA that might be of functional interest at the interface between the nervous system and the immune system. In this context several hitherto unknown DNA sequences were amplified from a human B-cell lymphoblast template and subsequently used to screen a corresponding cDNA library to obtain the full-length sequence. One such full-length cDNA, described in the present paper, turned out to have a particularly high degree of homology with the family of G-protein-coupled receptors activated by chemoattractant cytokines, the model substance being interleukin-8 (Holmes *et al.*, 1991). In accordance with the Genome Database Nomenclature rules the receptor was designated *CMKRL1*, which stands for "chemoattractant receptor-like 1."

MATERIALS AND METHODS

Isolation of cDNA clone. A cDNA library of a human B-cell lymphoblast cell line (GM03299; NIGMS Human Genetic Mutant Cell Repository, Camden, NJ) was constructed from poly(A)⁺-selected RNA in the pcD/SP6/T7 cloning and expression vector (Morel *et al.*, 1992), a derivative of Okayama-Berg's pcD vector (Okayama *et al.*, 1987). The library contained 7.5×10^6 recombinants. Plasmid preparation was prepared by cesium chloride-ethidium bromide banding (Sambrook *et al.*, 1989) and used as template (1 μ g) in PCR (Mullis and Faloona, 1987) attempting to amplify a DNA stretch between the putative TMII and TMVI of G-protein-coupled receptors. The sense primer was a 27-mer oligonucleotide with 250-fold degeneracy (5'-A(T)TCCTGGTG(C)A(T)G(A)CCTT(G)GCT(A)G(T)TGGCC(T)GAC-3'); the antisense primer was a 29-mer oligonucleotide with 128-fold degeneracy (5'-AT(G)GA(T)AGA(T)AGGGCAGCCAGC-

Sequence data from this article have been deposited with the EMBL/GenBank/DBJ Data Libraries under Accession No. X98356.

¹ To whom correspondence should be addressed at Section of Molecular Neurobiology, Department of Physiology and Neuroscience, Wallenberg Neuroscience Center, University of Lund, Solvegatan 17, S-22362 Lund, Sweden. Telephone: 46-46-222 0571. Fax: 46-46-222 0567.

AGAC(G)C(G)G(A)T(C)GAA-3'). The primers were used in 1 μ M concentrations together with *Taq* polymerase (Geneamp; Perkin-Elmer Cetus). Forty cycles of 96°C for 45 s (denaturation), 55°C for 4 min (annealing), and 72°C for 4 min (extension) were carried out, followed by a final extension at 72°C for 15 min. The products were analyzed on a 3% NuSieve genetic technology-grade agarose gel (FMC Bio-Products). Three bands between 500 and 700 bp in size were excised and blunted with *T4* polymerase, and terminal phosphates were added with *T4* polynucleotide kinase (New England Biolabs). The fragments were subcloned into the *HincII* site of the M13mp18 vector and sequenced according to Sanger's dideoxynucleotide termination method. Several sequences exhibited homology with the G-protein-coupled superfamily. Sequence information from one insert (hLym10) was utilized to obtain a full-length cDNA clone.

Screening of cDNA library. On the basis of sequence stretches in the PCR clone corresponding to the putative first extracellular and third intracellular loops, two 48-bp oligonucleotides were synthesized, one designated Lym5, 5'-ACACAGGAGGCAACCAGCCAG-TCCAAACTCCAGGTGCCTTGGGCCAG-3', and the other Lym6, 5'-GATCGGTGCCAGCACCCGCCGCCATCGCCTTGGTGCG-TAGCTTCTG-3'. They were labeled with [γ -³²P]ATP (5000 Ci/mmol, Amersham) and used in combination as probes to screen pools of recombinants prepared from consecutive dilutions (Bonner *et al.*, 1987) of the human B-cell lymphoblast cDNA library. Hybridization of Southern blots was performed in 3 \times SSC (0.45 M NaCl, 0.05 M sodium citrate, pH 7.0) at 60°C, and the filters were washed in 1 \times SSC at the same temperature. A positive band of 1.7 kb in size was followed until a single clone (designated Lym21-9) was obtained. Overlapping restriction fragments were subcloned into M13 phage vectors for sequencing of both cDNA strands. Sequence analysis and comparisons were performed with Genetics Computer Group software (University of Wisconsin) and with GenBank as well as with the GeneWorks program from IntelliGenetics (Mountain View, CA). Hydrophobicity tests of the deduced amino acid sequence were carried out according to Kyte and Doolittle (1982). Chromosome mapping results were evaluated in the Genome Data Base (GDB 6.0) and the NCBI database (Online Mendelian Inheritance in Man; OMIM).

Chromosome mapping. The procedures followed were those of Heng and Tsui (1993, 1994). Lymphocytes isolated from human cord blood were cultured in α -MEM medium supplemented with 10% fetal calf serum and phytohemagglutinin at 37°C for 68–72 h. The cultures were treated with 5-bromo-2'-deoxyuridine (0.18 mg/ml, Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37°C for 6 h in α -MEM with thymidine (2.5 μ g/ml, Sigma). Cells were harvested and suspensions dropped on slides using standard procedures including hypotonic treatment, fixation, and air-drying.

The probe used consisted of the entire plasmid construct of pcD vector including the Lym21-9 cDNA insert. It was biotinylated with dATP at 15°C for 2 h using the BioNick labeling kit (BRL).

For detection with fluorescence *in situ* hybridization (FISH) the slides were baked at 55°C for 1 h. After RNase A treatment the cell spreads were denatured in 70% formamide in 2 \times SSC for 1 min at 70°C followed by dehydration with ethanol. The biotinylated DNA probe was denatured at 75°C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulfate and human cot I DNA (BRL), and prehybridized for 15 min at 37°C. The probe mix was loaded on the denatured slides and hybridized overnight. The slides were washed and the hybridization signal was recorded in a fluorescence microscope, photographed, and amplified as described (Heng and Tsui, 1994). The assignment of FISH mapping data with a chromosomal banding pattern following staining with 4'-6-diamidino-2-phenylindole (DAPI) was achieved by superimposing photographic images according to Heng and Tsui (1993).

Cell transfection. Intact plasmid DNA (10–25 μ g) was used to transfect two cell lines: Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, MD) and mouse LVIP2.0Zc cells (König *et al.*, 1991), which contain a stably integrated fusion gene, pVIP2.0Z plasmid (Riabowol *et al.*, 1988),

consisting of the *Escherichia coli* LacZ gene under the transcriptional control of a fragment derived from the human vasoactive intestinal polypeptide (VIP) gene. The calcium phosphate precipitation method (Chen and Okayama, 1987) was used, and the pcD_{neo} vector was cotransfected to allow for selection with the neomycin analog, G-418 (Geneticin, 500 μ g/ml), which was started 72 h after the transfection and onward. The L cells were already maintained in the presence of 25 μ g/ml hygromycin as a selectable marker for phyg—a plasmid encoding hygromycin B phospho-transferase (Sugden *et al.*, 1985)—which had originally been cotransfected along with the VIP reporter construct. Monoclonal lines expressing the Lym21-9 cDNA were obtained by limiting dilution. Expression of the corresponding mRNA was verified by Northern blot hybridization with the same probes (Lym5 and Lym6) as used in the original library screening. Sham-transfected cells were used as controls.

Measurement of adenylyl cyclase activity. This was assayed in transfected whole cells and based on changes in cAMP levels using two approaches. One was a semiquantitative screening of candidate receptor ligands utilizing the mouse L cells. These were seeded into 96-well microtiter plates at 5×10^4 to 10×10^4 cells per well in 100 ml medium and incubated for an additional 24 h. Then another 100 ml medium was added containing the phosphodiesterase inhibitor, isobutyl methyl-xanthine (IBMX), and the test ligand. Ligands were assayed for inhibition of adenylyl cyclase in the presence of 1 μ M forskolin. The chromogenic substrate, *o*-nitrophenyl- β -D-galactopyranoside (ONPG, Research Organics), was added and any color change was measured in a plate reader (Molecular Devices) at 405 nm wavelength. In the other method, cAMP accumulation was determined quantitatively in radioimmunoassays using [¹²⁵I]-succinyl-cAMP as tracer (Brooker *et al.*, 1979). CHO cells were seeded in 24-well clusters at a density of 10^6 cells per well and grown to confluence. The growth medium was replaced with 250 ml medium containing the test agents and 1 μ M IBMX. Forskolin (1 μ M) was added to allow for recording of any inhibitory responses. The reaction was stopped after 5 min with 250 ml ice-cold solution containing 0.1 N HCl and 1 mM CaCl₂. Radioactivity was measured in a gamma counter with 85% counting efficiency.

Intracellular calcium measurements. Transfected and nontransfected cells were seeded on 30-mm-diameter glass coverslips and grown for 1–2 days to 40–95% confluence. They were loaded with 2 μ M Fura-2 in 500 μ l culture medium for 30 min at 37°C in the presence of 2.5 mM probenecid. After three washes in extracellular medium buffer (135 mM NaCl, 4.6 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 11 mM glucose, 11 mM Hepes, pH 7.4) the coverslips were mounted in a custom-made perfusion chamber on the stage of a Nikon inverted fluorescence microscope fitted to a photometer (Photon Technology International, Model D104). Measurements were carried out in extracellular medium buffer at room temperature, and the test agonists were added with a micropipette. Fluorescence images were obtained by alternate excitation at 340 and 380 nm, and the emitted light was measured at 510 nm. The slit was adjusted to allow for measurements from a single cell.

Tissue distribution of receptor mRNA. Northern blot hybridization was carried out according to the manufacturer's protocol (except for the use of 60°C washing temperature at 0.1 \times SSC) on premade nitrocellulose multiple tissue filters from Clontech (MTN Blot I and II, HCCL) covering 28 human tissues and cell lines. The hybridization probe consisted of isolated and phenol-purified full-length DNA of the Lym21-9 clone labeled by random priming (Megaprime, Amersham) with [α -³²P]CTP (3000 Ci/mmol, Amersham). All blots were exposed to Kodak XAR film with intensifying screens at -70°C for 4 days.

RESULTS

PCR with degenerate oligonucleotide primers homologous to DNA corresponding to the TMII and TMVI regions of several known neurotransmitter receptors resulted in the amplification of a 430 bp sequence

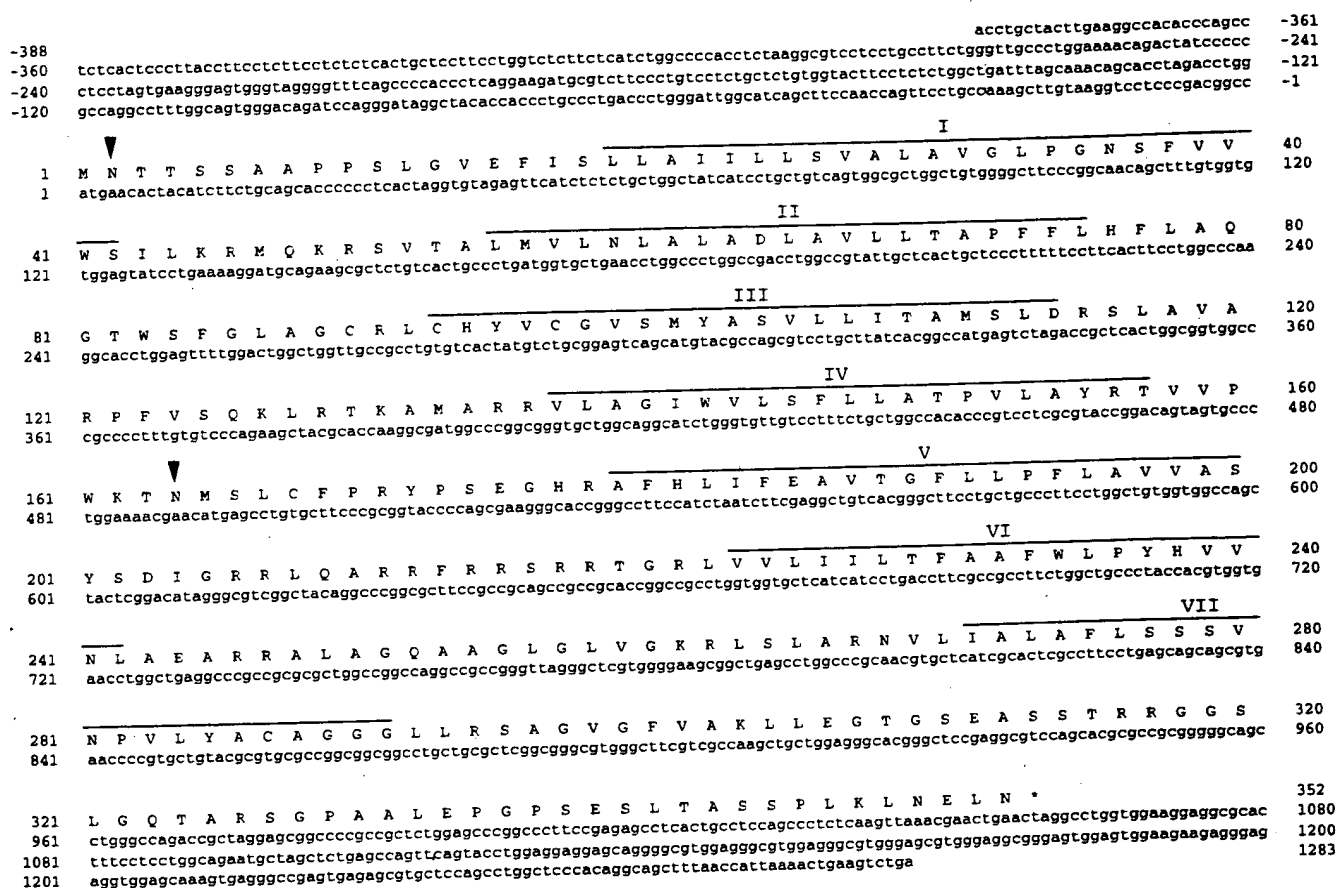


FIG. 1. Nucleotide sequence of the cDNA clone Lyme21-9 and the deduced amino acid sequence of the corresponding human CMKRL1 receptor. The first nucleotide and amino acid residue of the translation start site are designated as position 1. The putative transmembrane segments TM1-TM7 are indicated by solid lines; the extension of each segment is estimated on the basis of the hydrophobicity profile (Fig. 2), and sequence alignment of other heptahelix receptors. Potential glycosylation sites are indicated with arrowheads.

(hLym10) whose deduced amino acid sequence showed homology with other G-protein-coupled receptors. Oligonucleotide probes (Lym5 and Lym6) derived from this sequence were used to screen a human B-lymphoblast library. The 1.7-kb cDNA clone isolated was found to contain an open reading frame, with a putative initiator methionine conforming to the optimal sequence for translation (Kozak, 1986) and an in-frame downstream stop signal, encoding a 352-amino-acid polypeptide having a calculated relative molecular weight of approximately 43 kDa. Figure 1 shows the nucleotide sequence of this clone (Lyme21-9) and the amino acid sequence deduced from the coding region of the cDNA. The hydrophobicity profile (Fig. 2) of the peptide sequence shows the presence of seven segments probably forming the membrane-spanning regions of the receptor, which, for reasons given further below, was named CMKRL1. The predicted amino acid sequence also possesses other features common to the superfamily of G-protein-coupled receptors: (i) consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr, where X is any amino acid) near the amino terminus (Asn-2) and in the second extracellular loop (Asn-164); (ii) a conserved cysteine residue in each of the first two extracellular loops (Cys-90 and Cys-168), providing

possibilities to form a disulfide bond that stabilizes the functional protein structure; (iii) proline residues in all transmembrane regions (except TMIII) thought to induce flexibility within the helix formations, and (iv) a carboxyl terminus with several serine and threonine residues (10/4) that could serve as substrate for serine/threonine protein kinases. The predicted third intracellular loop, thought to be involved in the coupling to G-proteins and varying most in length among the different receptors, consists of only 21 amino acid residues.

Sequence comparison with cloned receptors within the G-protein-linked superfamily showed most similarity with the subfamily of chemoattractant leukocyte receptors (Fig. 3), particularly the "classical" chemoattractants, C5a and N-formyl-methionyl-leucyl-phenyl-alanine (fMLP) (Fig. 4). For example, there is (in the GCG/fasta matrix score) an approximately 30% overall sequence identity with the human C5a anaphylatoxin receptor (Gerard and Gerard, 1991) and 28% identity with the fMLP receptor (Boulay *et al.*, 1990; Thomas *et al.*, 1990). Taken as a group together with the Lyme21-9 receptor cDNA clone (Fig. 3), there is a particularly high degree of consensus in the GN-LVVLV sequence motif in the TM1 region and the LLNLA-DLLF-TLP-W motif within TMII.

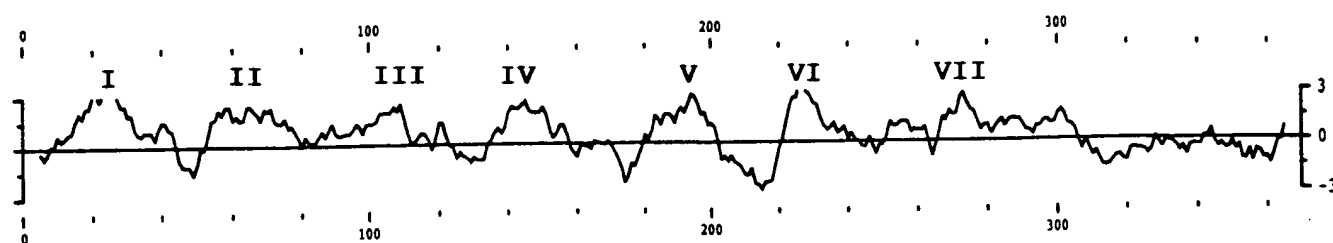


FIG. 2. Hydrophobicity pattern of the deduced amino acid sequence of Lyme21-9 according to the algorithm of Kyte and Doolittle (1982). The horizontal figures show the amino acid numbers. Regions (indicated positive) containing the maximally hydrophobic amino acid residues (over a window of nine in the standard GCG program) are numbered (I–VII).

The hybridization efficiency in the chromosome mapping with FISH was 68% (i.e., among 100 checked mitotic figures 68 showed signals on one pair of the chromosome). Based on the DAPI banding an assignment was obtained between the fluorescent signal from the Lyme21-9 DNA probe and the long arm of chromosome 14 (Fig. 5). The detailed position was further determined as a summation from 10 photomicrographs, indicating localization of the corresponding gene to region q11.2–q12 (Fig. 6).

The tissue distribution of the receptor in terms of message established in Northern blot hybridization was analyzed with the use of a full-length, gene-specific cDNA probe at high stringency. As shown in Fig. 7 the hybridization revealed two primary transcripts of slightly more than 5 and 7.5 kb in spleen, thymus, lymph nodes, bone marrow (large transcript predominating), and peripheral blood leukocytes (small transcript predominating) with the above-mentioned order of autoradiographic signal intensity. The two transcripts, alone or together, were expressed in some human cancer cell lines (Fig. 7): promyelocytic leukemia HL-60, lymphoblastic leukemia MOLT-4, Burkitt lymphoma Raji, lung carcinoma A549, and chronic myelogenous leukemia K-562. Some tissues—skeletal muscle, pancreas, and heart—showed only one, relatively weak, hybridizing band that was of smaller size, about 3 kb (Fig. 7). Two smaller bands appeared in the bone marrow, and they were seen at a lower signal intensity also in peripheral leukocytes. The tissue distribution of the message was corroborated in RT-PCR experiments (unpublished data) on normal human tissue. Hybridization with a structurally related but different cDNA probe (the Burkitt lymphoma receptor clone mentioned under Discussion) showed an entirely different hybridization pattern (unpublished data). Since the receptor is structurally similar to the chemoattractant receptors, particularly the “classical” ones, but also the C–C or β -chemokine receptors, it was named CMKRL1 (which stands for “chemoattractant receptor-like 1,” in conformity with the Genome Database Nomenclature rules).

Both L-VIP reporter cells and CHO cells were transfected with the Lyme21-9 plasmid, and permanently expressing clones of both cell lines were successfully established as revealed by positive bands appearing in Northern blot hybridization with the full-length cDNA

(Lyme21-9) probe. Extensive attempts have been made to activate the exogenous CMKRL1 receptor with a large number of putative ligands, including the chemotactic peptides GRO- α , MCP-1, MCP-3, MIP-1a, MIP-1b, C5a, RANTES, and LTB $_4$ (all used at a concentration up to 100 μ M), as well as conditioned medium from cell lines known to produce cytokines, but results have so far been negative in measurements of both increases and decreases in cAMP levels, as well as cellular calcium fluxes measured by Fura-2 fluorescence.

DISCUSSION

A full-length cDNA (Lyme21-9) encoding a novel putative seven-transmembrane (7TM) domain receptor, named CMKRL1, was identified in a human B-cell lymphoblast library by screening with degenerate primers and radiolabeled oligonucleotide probes corresponding to neurotransmitter receptors. Highest expression of receptor message was found in leukocytes, lymph nodes, thymus, and bone marrow. Northern blot hybridization with a specific, full-length cDNA probe showed—even under high stringency conditions—several bands with sizes varying between approximately 7.5 and 1.5 kb. Only the smallest bands corresponded to the size of the cloned cDNA. There could be several reasons for this, given that the hybridization signal is specific. There may be splice variants of the message and/or a varying expression of different entities in the course of cell maturation. Indeed, several potential splice sites, identified by, e.g., the base combination CAGG (cf. Mattaj and Séraphin, 1992), can be seen in the currently cloned cDNA sequence. The cDNA may have been synthesized primarily from the short mRNA individuals, or even from truncated forms, in the construction of the cDNA library (Okayama *et al.*, 1987).

While many cloned G-protein-linked receptors belong to the neurotransmitter receptor category, only relatively few are specifically associated with the immune system (e.g., Murphy, 1994; Watson and Arkin-stall, 1994; Kunkel *et al.*, 1995). Several novel receptor-DNA sequences that may belong to this functional category based solely on predicted amino acid similarity have been cloned during the past few years, though the ligands involved have not yet been identified. Members of this receptor family are the BLRs, expressed in Burkitt lymphoma and in lymphocyte cell lineages (Dobner

humL8A	1	MSNITDPOMWDEDDL	...	FTGMPADEDSYSCMLETETLNKYVVI	IAYA	...	LVFLLSLL	...	GNLSVMLVILYSRVGRSV	TDVLLNL	...	ADL	87	
humL8B	1	MESDSFEDFWKGEDLS	...	SYSSSTLPELLDAAPCEPESLE	INKYFVVI	IYA	...	LVFLLSLL	...	GNLSVMLVILYSRVGRSV	TDVLLNL	...	ADL	91
humCKR1	1	METPNITTE	...	DYDTITTEFDYGDATPCQKVN	ERAFGAQL	PPLYS	...	LVFVIGLV	...	GNLSVMLVILYSRVGRSV	TDVLLNL	...	ADL	82
humMIP1aR	1	PEPMEIR	...	INTINESGEEVITTFEDYDYGAP	CHKFKDVKQ	IGAQLPPLYS	...	LVFVIGLV	...	GNLSVMLVILYSRVGRSV	TDVLLNL	...	ADL	88
humCKR2	1	MLSTSRSR	...	IRINTINESGEEVITTFEDYDYGAP	CHKFKDVKQ	IGAQLPPLYS	...	LVFVIGLV	...	GNLSVMLVILYSRVGRSV	TDVLLNL	...	ADL	90
humMCP1r	1	PEPMLSTSRSR	...	IRINTINESGEEVITTFEDYDYGAP	CHKFKDVKQ	IGAQLPPLYS	...	LVFVIGLV	...	GNLSVMLVILYSRVGRSV	TDVLLNL	...	ADL	96
humC5AR	1	MNSFN	...	YTPDYGHYDDKDLTDLN	ISGVT	84
humMFLR	1	73
Lyme21-9	1	66
humL8A	88	FALTLP	...	PIWA	...	ASKVNGWI	...	FGTFLCKVVSLL	...	KEVNFYS	GI	172
humL8B	92	FALTLP	...	PIWA	...	ASKVNGWI	...	FGTFLCKVVSLL	...	KEVNFYS	GI	176
humCKR1	83	FLFTLP	...	FWI	...	DYKLDKDDWV	...	FGDAMCKILSGFY	...	YTGLEYSE	IF	169
humMIP1aR	89	FLFTLP	...	FWI	...	DYKLDKDDWV	...	FGDAMCKILSGFY	...	YTGLEYSE	IF	181
humCKR2	91	FLITLP	...	WAH	...	SAANEV	...	FGNAMCKLFTGLYH	...	GYEGG	IF	176
humMCP1r	97	FLITLP	...	WAH	...	SAANEV	...	FGNAMCKLFTGLYH	...	GYEGG	IF	186
humC5AR	85	SCALP	...	ILFTS	...	VOHHH	...	WPFGGAA	...	CSILPS	172
humMFLR	74	FTSTLP	...	FFMVRKAMGGH	WPFGGAA	...	CSILPS	181
Lyme21-9	67	VLLTAP	...	FLHFLAOGT	WSFGLAG	...	CHYVCGVSMYASVLL	153
humL8A	173	LFR	...	QA	...	YHPNNS	...	PVCYEVLMG	...	NDTAKWR	258
humL8B	177	LFR	...	QA	...	YHPNNS	...	PVCYEVLMG	...	NDTAKWR	260
humCKR1	170	YFS	...	KTOWE	...	THHTCSLH	...	FPHE	...	SLREWK	253
humMIP1aR	177	YFS	...	KTOWE	...	THHTCSLH	...	FPHE	...	SLREWK	257
humCKR2	177	IFT	...	KCOKE	...	DSVYVCGPYFP	275
humMCP1r	177	IFT	...	KCOKE	...	DSVYVCGPYFP	275
humC5AR	173	LYRV	...	AREEYFP	...	PKVLGVDYSH	256
humMFLR	162	L	...	RVTPGKT	...	GTACTFNSP	...	WTNDPKERIN	...	AVAMLT	255
Lyme21-9	154	AVRTV	...	VPWKTNMS	...	LCFPRYPSEGR	235
humL8A	267	PYNVLL	...	ADTL	...	LMRTQVIOET	...	GER	...	RNNIGRAL	...	DATEIL	...	328
humL8B	267	PYNVLL	...	ADTL	...	LMRTQVIOET	...	GER	...	RNNIGRAL	...	DATEIL	...	328
humCKR1	254	PYNLT	...	ILSV	...	FODLFTHE	...	CEO	...	SRHLDL	...	AVQTEVIA	...	332
humMIP1aR	272	PYNLT	...	ILSV	...	FODLFTHE	...	CEO	...	SRHLDL	...	AVQTEVIA	...	332
humCKR2	259	PYNIV	...	ILLNT	...	FOEFFGLSN	...	CEO	...	TSQDQ	...	QATVETEL	...	357
humMCP1r	278	PYNIV	...	ILLNT	...	FOEFFGLSN	...	CEO	...	TSQDQ	...	QATVETEL	...	357
humC5AR	257	PYOVT	...	GIM	...	MSFLEPSS	...	PTFLNKL	...	SLC	343
humMFLR	256	PYQV	...	VALI	...	ATVRI	...	RELQGM	...	YKEIG	...	IAVDVT	...	343
Lyme21-9	236	PYHV	...	VNLA	...	EARRAL	...	AGQAAGL	...	GLQGL	...	VGKRLS	...	329
humL8A	329	EFLAR	...	HVRTSY	...	TSSSVNVSSNL	350
humL8B	333	DSLP	...	KDSRPS	...	FVGSSTGHTSTTL	355
humCKR1	337	ERSV	...	STSPSTGEHE	...	LSAGF	355
humMIP1aR	358	DRVST	...	STSPSTGEHE	...	PEPLSAGF	379
humCKR2	352	DGRG	...	KGKS	...	IGRAPEASLQDK	...	KEGA	374
humMCP1r	362	DGVST	...	NTPTSP	...	PEPTGEDEV	...	SAGL	384
humC5AR	344	EVAL	...	QAK	350
humMFLR	344	AALE	...	PGPSES	...	LASSPLKLNEL	351
Lyme21-9	330	AALE	...	PGPSES	...	LASSPLKLNEL	352

FIG. 3. Alignment of the complete amino acid sequences for eight human chemotactic receptors together with the amino acid sequence deduced from the presently cloned cDNA (Lyme21-9), showing the high degree of similarity (shaded areas), not least within the transmembrane regions. The homology presentation was done with the SeqVu (version 1) mode in the GCG program. The scaling system used is described by Riek *et al.* (1995).

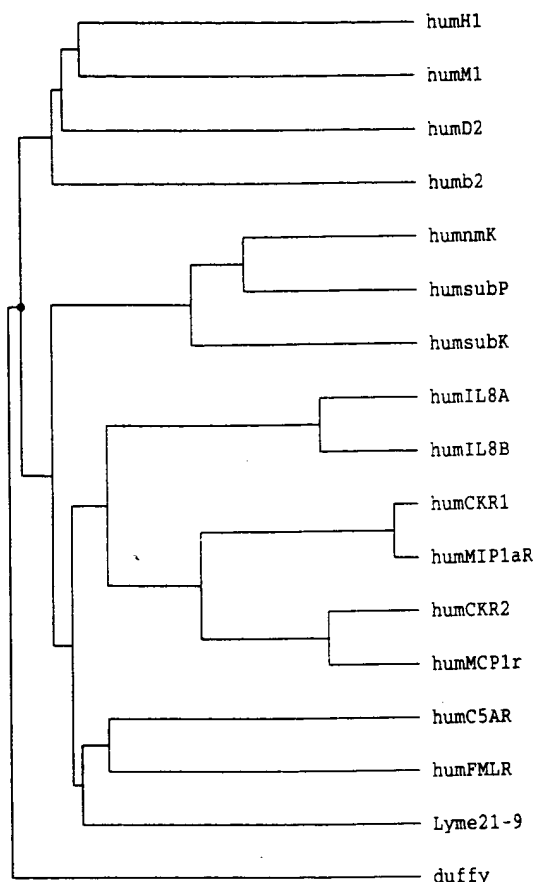


FIG. 4. Dendrogram (the horizontal distances to the branching points corresponding to the relative degree of sequence identities) based on the eight human chemoattractant receptors listed in Fig. 3 as well as the Duffy antigen, together with the currently cloned Lyme21-9, showing similarities in the evolutionary pattern. For comparison, the dendrogram also includes a selection of "unrelated" human receptors belonging to the rhodopsin-type family: the amine receptors histamine H1, muscarinic M1, dopamine D₂, adrenergic β_2 , and the peptide receptors neuromedin K, substance P, and substance K. Full amino acid sequences were used in the multiple alignments and the dendrogram and they were performed with the PileUp software in the GCG program.

et al., 1992). We recently cloned (unpublished results) another heptahelix-type Burkitt lymphoma receptor from the same B-lymphoblast library used in the present study. The peptide sequence deduced from that cDNA shows, however, only approximately 22% identity with the corresponding sequence of Lyme21-9, and while ubiquitously distributed, there is no expression in peripheral blood leukocytes, in contrast to the situation for Lyme21-9. Recently, another 7TM receptor expressed in leukocytes, LESTR, was cloned from a monocyte library (Loetscher *et al.*, 1994) and was found to be the human equivalent of the bovine brain boLCR1, which was mistakenly thought to be a neuropeptide Y (NPY) receptor (Rimland *et al.*, 1991). A cDNA sequence with more than 90% amino acid identity to the bovine sequence has been cloned from human lung and kidney libraries, and the corresponding gene was found to reside in chromosome 2 (Herzog *et al.*, 1993). Strong

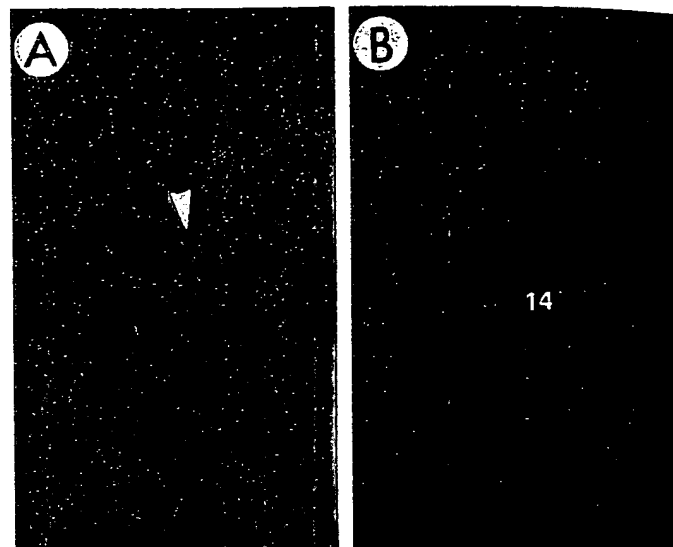


FIG. 5. Fluorescence photomicrographs with examples of FISH mapping of the gene corresponding to CMKRL1. (A) The fluorescent signals on one human chromosome. (B) The same mitotic figures stained with DAPI to identify chromosome 14. Original magnification $\times 1300$.

attention has recently been given to these receptors because they seem to be an entry-cofactor for HIV-1, and are hence termed "fusins" (Feng *et al.*, 1996), in CD4-positive target cells. Another lymphoid-specific receptor, EBI1, originally identified as an Epstein-Barr-induced cDNA (Birkenbach *et al.*, 1993), has turned out to be expressed also in normal lymphoid tissues; the corresponding gene is located in human chromosome 17 (Schweickart *et al.*, 1994). The IL-8 receptor genes are clustered on human chromosome 2 (Ahuja *et al.*, 1992). The present Lyme21-9 gene was found to have yet another localization—on chromosome 14. The chemo-

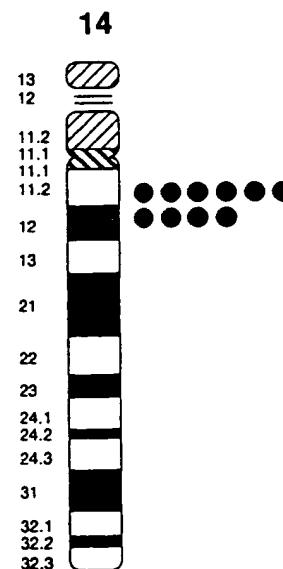


FIG. 6. Diagram of FISH/DAPI mapping results. Each dot represents the double fluorescent signals detected on chromosome 14 (images from 10 photographs).

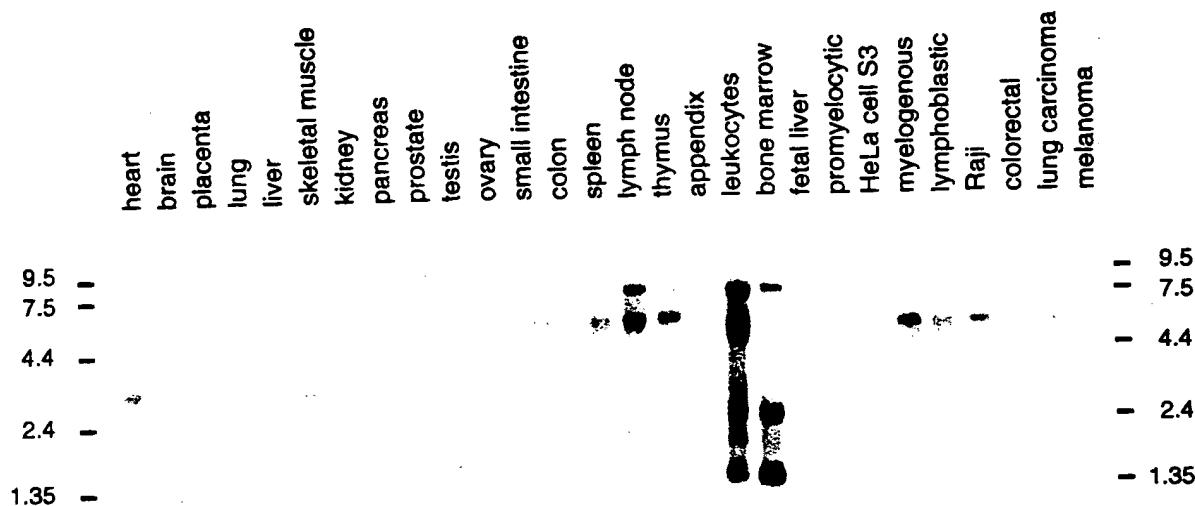


FIG. 7. Northern blot hybridization of mRNA (2 mg poly(A)⁺ RNA per lane) from 28 human tissues and cells with the Lyme21-9 cDNA probe. Constantly appearing hybridizing transcripts are approximately 5 and 7.5 kb (RNA molecular size markers are shown on both sides), appearing alone or in pairs, in addition to a 3-kb transcript, appearing alone in some tissues. Yet smaller bands are seen in bone marrow and with lower signal intensity also in leukocytes. A detailed account of the the distribution of the transcripts appears in the text. The cancer cell lines are promyelocytic leukemia HL-60, HeLa cells S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361.

kine-like human receptor gene, R2, has recently been mapped to this chromosome as well (Raport *et al.*, 1996). Thus, there seems to be no common denominator in this context for a clue to receptor identification. Indeed, a search in the Genome Data Base did not provide any further clues about possible ligands or any disease of interest mapped in human chromosomes to the particular region in which the CMKRL1 gene is localized.

Because of the similarity to chemoattractant receptors and the high expression in lymphoid tissues including leukocytes, the currently cloned receptor was designated CMKRL1. Murphy (1994) has listed five consensus features that appear to identify chemoattractant receptors as a distinct subfamily among the heptahelix receptors. Thus, their sequences are similar in length, approximately 350 amino acid residues (CMKRL1 is 352 residues long), which places them among the smallest in the receptor superfamily. In addition, they show around 30% overall identity (the same as CMKRL1) in their peptide sequence. The third intracellular loop is short (16–22 amino acid residues; the deduced peptide of CMKRL1 has 21 residues) and is enriched in basic amino acids. In contrast, the N-terminal segments are usually acidic in the chemoattractant receptors, like the currently cloned receptor. Finally, a common characteristic of the receptors is expression of their messages in leukocytes.

There may be several reasons why attempts to define functionally the ligands that bind to the above-mentioned lymphoid-specific "orphan" receptors have been negative. Apparent reasons include the problem of appropriate translation of the exogenous receptor transcript in transfected cell lines and inappropriate coupling or lack of coupling to second messenger. Indeed, the particular second messenger system involved in the

transduction of any of the unknown receptors may need to be defined as well; the chemokine-type receptors may be expected to transduce through inositol phosphate metabolism and calcium fluxes (Murphy, 1994). However, the human fMLP receptor also requires for its intracellular signaling a complementary factor that is not identical to the α subunit of the heterotrimeric G proteins G_{11} , G_{12} , or G_{13} (Murphy and McDermott, 1991). Cloning of CMKRL1 showing identity with chemoattractant-type receptors extends the growing list of lymphocyte-expressing G-protein-coupled receptors that may be involved in the control of lineage-specific stages during B-cell lymphopoiesis (cf. Kincade, 1994) or may be involved in inflammation as regulators of the lymphocyte trafficking function. (Förster *et al.*, 1994).

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